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ORIGINAL PAPER

Designing a multi-species inoculant of phosphate rock-solubilizing bacteria compatible with arbuscular mycorrhizae for plant growth promotion in low-P soil amended with PR

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Abstract

Four phosphate rock-solubilizing bacteria (PRSB) (*Asaia lannensis* Vb1, *Pseudomonas* sp. Vr14, *Rahnella* sp. Sr24, and *Pantoea* sp. Vr25) isolated from the mycorrhizosphere of maize were evaluated as inoculants, individually or in different combinations, together with *Rhizophagus irregularis* DAOM 197198, under greenhouse conditions. To create an effective bacterial mix for P solubilization and growth promotion, parameters such as the ability of the strains (individually or in mixture) to mobilize phosphate rock (PR), their biocompatibility, and their capacity for biofilm formation over PR particles and root colonization were tested. Accompanying PR solubilization, the bacteria produced organic acids and reduced the pH of the medium, produced exopolysaccharides, and had variable capacity to colonize the roots of maize plantlets. In low-phosphorus soil amendment with PR, all strains, regardless of their capacity to solubilize PR, increased dry weight, nutrient (N, P, and K) uptake, and the percentage of indigenous arbuscular mycorrhizae (iAM) root colonization in maize plants, compared to the non-inoculated control. However, mixed inoculation of the strains showed significantly better results. Addition of *Rhizophagus irregularis* resulted in further growth stimulation. Overall results showed that two combinations—*Rahnella* sp. Sr24 + *Pantoea* sp. Vr25 + *R. irregularis*, and *Pantoea* sp. Vr25 + *Pseudomonas* sp. Vr14 + *R. irregularis*—were better inoculants. We concluded that an effective PRSB combination of biocompatible strains with capacity for PR solubilization, successful biofilm formation, effective root colonization, different growth promotion traits, and the addition of AM has potential as inoculants for more sustainable agriculture in low-phosphorus soils amended with low-reactivity PR.

Keywords Arbuscular mycorrhizae · Rhizobacteria · Rock phosphate · Biofilm · Root colonization

Introduction

Phosphorus (P) is one of the most important nutrients for plants but it is a limited resource (Hinsinger et al. 2011). The world's reserves of high grade P ores are decreasing, but P demand is expected to increase globally (Van Vuuren et al. 2010). Rising global food production demands every effort to make P availability for plants more efficient through the use of better use of soil P reserves, direct use of low grade P ores (like phosphate rock [PR]), and a reduction in the use of expensive chemical fertilizers. One well-known option to increase P availability in soils is the use of phosphate-solubilizing microorganisms (PSMs) as inoculants. These PSMs, such as PR-solubilizing bacteria (PRSBs) and arbuscular mycorrhizae (AM) fungi, can be used for sustainable agriculture, especially when low-solubility P sources, such as rock phosphate, are

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used as fertilizers (Hameeda et al. 2008). Additionally, PSMs can exploit important but scarce nutrients already present in the soil (P soil reserves), thereby improving sustainable agriculture in low-P soils and leading toward higher ecological stability and environmental quality (Khan et al. 2007).

Microbial involvement in the solubilization of sparingly soluble P sources is well documented. However, most of the studies deal with the characterization of PSM's ability to dissolve P under in vitro conditions as a separate characteristic of other plant growth–promoting rhizobacteria (PGPR), such as the production of indoleacetic acid, siderophores, compatibility with arbuscular mycorrhizae, biofilm forming capacity, and root-colonization capacity. Because the studies underscore the general capacity of PSMs to enhance P availability for plant growth, a major limitation of the research is a lack of consistency in observing the effects of mobilizing P under field conditions due to interference by the native microflora, environmental factors (Martinez-Viveros et al. 2010), and P soil concentration. Thus, recent studies have focused on the co-inoculation of different PSMs to foster synergistic interactions and therefore obtain better results than those of single inoculation treatments (Khan et al. 2007). Increasingly, the diversity of PSM in an inoculant ensures the bacterial capacity to occupy different niches and to enhance the positive effect of the inoculation. The use of microbial consortia instead of one single strain can provide multiple benefits, including the occupation of different ecological niches (Pandey et al. 2012). Also, combining plant growth promotion with bioremediation (such as exploiting sparingly P sources unavailable in the soil) through the design of a microbial consortia could lead to a beneficial approach to address the use of chemical fertilizers (Backer et al. 2018).

It is reported that AM in combination with some PGPR have the potential to enhance plant P uptake and to act synergistically to improve plant nutrition (Miransari 2011). However, the interaction between AM and rhizobacteria can be affected by soil conditions, such as mechanical disturbances or water stress (Mickan et al. 2019). Furthermore, it has been recognized that N and P application affects the abundance of AM (Xiao et al. 2019); thus, it is relevant to consider the soil factor when establishing an interaction between AM and rhizobacteria. In agricultural soils, it is recognized that some P-solubilizing bacteria also interact with AM to increase plant growth (Barea et al. 2005; Mäder et al. 2011). Mixed inoculation with diazotrophic bacteria and AM fungi frequently creates synergistic interactions that increase plant and N and P nutrition (Bashan 1998; Taktek et al. 2015, 2017; Ordoñez et al. 2016). Furthermore, combining microbial inoculation of PSMs with PR application is generally effective in improving the sustainable nutrient supply for plants (Toro et al. 1997; Battini et al. 2017). In rural regions, where low-input agricultural systems occur, natural AM co-inoculated with PGPRs can substantially improve the production of important crops such as wheat (Mäder et al. 2011) and maize (Wu et al. 2005).

In a more recent work, Khan et al. (2019) developed an artificial consortium of *Bacillus* sp. with different capacities to produce indoleacetic acid (IAA) and extracellular polysaccharides, which play an important role in protecting the plant from desiccation, in order to enhance the capacity of chickpea to tolerate drought. They observed that mixing different PGPR and plant growth regulators (PGR) had a beneficial synergic effect on the plants. However, the authors did not test the biocompatibility between the strains. Multi-species consortiums, such as the *Bacillus subtilis* SM21, *B. cereus* AR 155, and *Serratia* sp. XY21 (BBS), have also been used for biocontrol against *Phytophthora* on sweet pepper disease (Zhang et al. 2019). Furthermore, Raklami et al. (2019) found an enhancement in the growth of bean and wheat under field conditions by using a consortium of PGPRs (with different P solubilization capacities, IAA, siderophores, hydrogen cyanide production, and nitrogen fixation) and AM. They proved that this inoculum was adapted to the soil's native microflora in open-field conditions and had an important effect on soil physicochemical properties.

The vast majority of these studies do not consider the compatibility between PSM strains, biocompatibility with AM, root colonization capacity, and other plant growth–promoting traits, such as biofilm formation and motility. The literature also rarely shows screening of PR-solubilization in vitro of all possible combinations of the strains to select those that provide the highest soluble P concentrations or the maximum PR weathering capacity. Thus, our hypotheses are the following: hypothesis 1, the formulation of a multi-species inoculant of PRSBs with different traits, besides phosphate solubilization capacity, will be more effective than monospecific inoculation, and hypothesis 2, biocompatibility of the bacterial inoculant with AM will improve plant P nutrition and growth under low-P soils when amended with low-reactive PR. The aim of the work was to design a multi-species and AM-biocompatible inoculant of PRSBs with different plant growth–promoting traits. Thus, we evaluated growth promotion of maize by the selected isolates individually or in different combinations with *Rhizophagus irregularis* under greenhouse conditions.

Material and methods

Selected isolates

The four bacteria selected for these studies were previously isolated from the mycorrhizosphere of maize. All strains proved to be compatible with *Rhizophagus irregularis* and were tested for different plant growth–promoting traits (Magallon-Servin et al. 2019). Their capacity to solubilize different sources of low-soluble phosphates (AlPO4, FePO4, hydroxyapatite, and three different PR [Gafsa, Tilemsi, and

Morocco]) was assessed as suggested by Bashan et al. (2013) (Magallon-Servin et al. 2019). The isolates were identified as *Asaia lannensis* Vb1 (GenBank KJ939699), *Pseudomonas* sp. Vr14 (GenBank KJ939703), *Rahnella* sp. Sr24 (GenBank KJ939705), and *Pantoea* sp. Vr25 (GenBank KJ939706). *A. lannensis* Vb1 is a high PR solubilizer, and *Pseudomonas* sp. Vr14, *Rahnella* sp. Sr24, and *Pantoea* sp. Vr25 are positive for IAA production, siderophores, alkaline, and acid phosphatases and phytase. Since PR contains different accessory minerals besides P, all strains were tested for their resistance to heavy metals through minimum inhibitory concentration evaluation (0.78 mM–100 mM of Fe, Al, Cu, Zn, and Mn) in different media (Supplementary Material Table S1). All strains were deposited in the microbial collection of Prof. Antoun at the Centre de Recherché en Horticulture of Laval University, Quebec, Canada. The AM fungus used here was *Rhizophagus irregularis* DAOM 197198 (PremierTech, Rivière-du-Loup, Quebec, Canada).

Biocompatibility assay

Biocompatibility among the four selected isolates was determined by cross-culturing the isolates in all possible combinations. For that purpose, a bacterial suspension (1×10^7 CFU mL^{-1}) of a particular isolate was spread on Trypticase Soy Agar (TSA) (Difco) using sterile glass beads, and the plates were incubated at 28 °C overnight. Then, another TSA plate was used to spread each target strain (three plates per isolate), and a 5-mm disk of TSA containing one active colony of each of the PRSBs was placed over the plate already containing the target strain. This procedure was done in triplicate. Plates were incubated overnight at 4 °C in order to allow diffusion of inhibitory substances. Finally, the plates were incubated at 28 °C for 24 h. The inhibitory halo over the target strain caused by any of the tested strains was registered.

PRSB inoculum preparation

The strains were cultured in 250-mL Erlenmeyer flasks containing 100 mL Trypticase Soy Broth (TSB) 10% (wt/vol) (Difco). Flasks were incubated overnight at 28 °C on a rotary shaker (150 rpm). Cells were harvested and washed twice in saline solution (NaCl 0.85% wt/vol) after centrifugation at 10,000×*g* for 15 min at 4 °C. The OD_{600nm} of the bacterial suspension (BS) was adjusted to 0.4, which corresponds approximately to 1×10^8 CFU (colony-forming units) mL^{-1} (Magallon-Servin et al. 2019). To prepare mixed inoculum, equal volumes of each strain were mixed to achieve 25 mL in a Falcon sterile tube.

Phosphate rock solubilization, pH, and organic acid production of the four strains, individually and in combination

The ability of the bacterial isolates to, individually and in combination, solubilize Moroccan 0:13:0 PR (McInnes Natural Fertilizers®, Quebec, Canada) (MPR) in National Botanical Research Institute's phosphate (NBRIP) growth medium (Nautiyal 1999) was determined. The MPR was washed twice with hot tap water to remove any soluble P, dried, and ground (to pass 0.130 mm–0.159 mm openings) prior to use (Reyes et al. 2001). Triplicate 25-mL volumes of medium containing 5 g L^{-1} of MPR (NBRIP-MPR) instead of $Ca_3(PO_4)_2$ were inoculated with 100 μL of inoculum (used individually or in mixture) in 50-mL Erlenmeyer flasks. Flasks were incubated on a rotary shaker at 150 rpm and 28 °C for 7 days. At the end of the experiment, aliquot samples (5 mL) were centrifuged at 10,000×*g* for 10 min at 4 °C and filtered through a 0.22-μm Millipore filter. The resulting supernatant was used to determine soluble P (Fiske and Subbarow 1925), pH, total protein (Fryer et al. 1986), and organic acid (OA) production. OAs were separated on a Waters high-performance liquid chromatography system (HPLC Pump Model 996; Waters Limited, Mississauga, Ontario, Canada) using a 300 × 7.8 mm 5-μm ICSep ICE-ION-300 column maintained at 40 °C in a column oven, with an isocratic solvent (8.5 μM H_2SO_4) at a flow of 0.4 ml·min^{-1}. OAs were detected by a photodiode array detector (Waters, Model 600) at 210 nm. The identification of individual OA peaks was performed by comparing retention times with standards (Sigma-Aldrich, Mississauga, Ontario, Canada).

Determination of biofilm formation capacity on abiotic surfaces and over PR surface

The biofilm formation by the PRSBs was determined by the Specific Biofilm Index (SBF), with the crystal violet method modified as described by Naves et al. (2008). For each strain, 10 μL of each isolate inoculum (1×10^8 CFU mL^{-1}) were added to a well containing 200 μL of TSB (Difco) 50% (wt/vol) in a 96-well cell culture cluster (flat bottom, tissue culture treated, nonpyrogenic, polystyrene Corning Costar). For each treatment, three replicates were prepared. The plates were incubated at 28 °C without agitation. Bacterial growth was determined by measuring OD_{620nm} with a microtiter plate reader, and 50 μL of a crystal violet solution 0.3% Difco BD (wt/vol) was added to each well and left for 15 min. Excess stain was removed by rinsing the wells at least four times with tap water. Plates were air-dried for 4 h before extraction of the crystal violet with 200 μL of 95% (vol/vol) ethanol overnight in a rotary shaker (60 rpm) (Barahona et al. 2010). After

incubation, OD_{540} nm was determined in a microplate plate reader and SBF was determined by following the formula of Naves et al. (2008):

$$SBF = ((AB-CW)/G)$$

where AB is the OD_{540nm} of the stained attached bacteria, CW is the OD_{540nm} of the stained control wells containing bacteria-free medium only, and G is the OD_{620nm} of cell growth in the suspended culture.

For analysis of biofilm formation over PR, bacterial inoculum was prepared as described earlier. In the procedure, 100 μL of each strain (individually or in mixture) was added to a well containing 8 mL of NBRIP-MPR medium in a flat bottom, six-well cell culture microplate (flat bottom, tissue culture treated, nonpyrogenic, polystyrene Corning Costar). For each treatment, three replicates were prepared. The six-well plate was incubated at 28 °C without agitation for 5 days. After incubation, a sample aliquot (5 mL) was carefully removed from each well with a pipette, collected in a 25-mL Falcon sterile tube and centrifuged at 10,000×*g* at 4 °C. Supernatant soluble P was quantified using the method described by Fiske and Subbarow (1925) adapted to a microplate, and pH was measured.

The biofilm was evaluated by quantifying the amount of extracellular polysaccharides, according to Dubois et al. (1956) and modified by Furtner et al. (2007). Each well was rinsed carefully twice with saline solution (NaCl, 0.85% wt/vol), then 4.42 mL of saline solution containing 0.23% (vol/vol) of formaldehyde (Sigma-Aldrich) was added. The plates were agitated at 80 rpm on a rotary shaker at room temperature for approximately 5 min and then incubated at 4 °C for 1 h. After incubation, 1.76 mL of a 1-N solution of NaOH was added and the plates were incubated for another 10 min. Finally, 1.5 mL of the sample was recovered in a 2-mL Eppendorf tube. The tubes were centrifuged at 10,000×*g* at 4 °C for 1 min. Then 800 μL of the supernatant was recovered in a 15-mL glass tube and mixed with 800 μL of a phenol 5% (vol/vol) solution and 4 mL of concentrated H_2SO_4. The mixture was cooled down and $OD_{488\ nm}$ was determined. The amount of extracellular polysaccharides was determined using a standard curve of D-glucose (0–0.2 mg L^{-1}).

Effect of the different PRSB strains on colonization of maize seedling roots

Maize (*Zea mays* L. cv Seneca Horizon) seeds were surface disinfected by soaking for 1 min in 70% ethanol and 5 min in commercial sodium hypochlorite (5.25%), followed by five rinses in sterile distilled water. Seeds were allowed to germinate in 9-cm Petri dishes containing water agar (15 g L^{-1}) in the dark for 48 h at 26 °C. The treatments consisted of each strain alone (*Asaia lannensis* Vb1, *Pseudomonas* sp. Vr14, *Rahnella* sp. Sr24, and *Pantoea* sp. Vr25), various bacterial combinations (Vb1+Vr14, Vb1+Vr25, Vb1+Sr24, Vb1+Vr14+Vr25, and Vr14+Sr24+Vr25), and a non-inoculated control. The specific combinations were selected because (a) Vr1 presents a high P solubilization in vitro condition, and (b) Vr14, Sr24, and Vr25 were previously proven to show PGP-associated traits (Magallon-Servin et al. 2019). The inoculum was prepared as described earlier. Uniformly germinated seeds were incubated at room temperature for 1 h in a 50-mL Falcon tube containing 20 mL of inoculum, with gentle agitation (60 rpm). Two inoculated seeds were planted in each sterile growth pouch (Seed Germination Pouch, 18 × 12.5 cm, PhytoAB) containing 10 mL of a sterile complete Hoagland solution. Seven growth pouches were placed vertically in metal racks, and for each treatment three racks were used. Treatments were placed in a growth chamber (Conviron) in a randomized design, with three replicates and a total of 42 seedlings per treatment. The growth chamber was adjusted to 25 °C, 12 h light/12 h dark, and 80% humidity. Pouches were watered every 2 days with 10 mL of Hoagland solution. After 2 weeks, plants were harvested, and dry weight was measured after 72 h at 65 °C.

To determine the capacity of the different isolates to colonize maize, 3 g of roots (fresh weight) from one growth pouch of each repetition per treatment was placed in a sterile Falcon tube containing 20 mL of saline solution (NaCl 0.85%, wt/vol). Tubes were then shaken carefully at 100 rpm for 60 min at room temperature, and a series of dilutions were performed in sterile saline solution (dilutions 10^{-4}–10^{-6}). Plates containing NBRIP media, supplemented with 5 g L^{-1} of hydroxyapatite (Sigma-Aldrich) instead of $Ca_3(PO_4)_2$ (NBRIP-Hxa) with 12.5 μg mL^{-1} of cycloheximide, were used for plate count. Plates were incubated at 28 °C for 72 h. Bacteria showing a solubilization halo on NBRIP-Hxa were counted as total count. However, the total count was divided by the individual counts of each isolate, which was achieved by observing the typical morphology of each strain considering form, shape, and color.

Effect of the strains on the growth of maize in the presence of *Rhizophagus irregularis* in low-P soils amended with PR: greenhouse experiment

Soil properties and experimental design

Soil was collected from Saint-Joseph-de-Beauce, Québec, Canada (46° 22′ 25″ N, 71° 01′ 55″ W). The soil was a sandy loam with a pH of 4.6 (1:1 in water) and a buffered pH of 5.5, determined by the Shoemaker–McLean–Pratt method (Shoemaker et al. 1961). Lime (15 t ha^{-1}) was added to increase the pH to 6.8. The soil characteristics were 5.1% organic matter, a cation-exchange capacity of 19.5 $cmol_c/kg$, P and Al Mehlich III kilograms per hectare of 16.8 and 4870.56,

respectively, giving a $(P/Al)_{MIII}$[%] value of 0.34 (P/Al_{MIII}[%] = [P_{M3} kg ha^{-1}/Al_{M3} kg ha^{-1}] × 100). The K, Mg, Ca, Zn, Mn, Cu, and Fe available in the soil, determined using ammonium acetate as the extracting solution, were 148.8, 165.5, 350.4, 6.96, 37.68, 3.12, and 403.44 kg ha^{-1}, respectively. A bifactorial complete randomized block design with 20 treatments and four replicates (two subsamples per replicate) was used. The 10 PRSB inoculation treatments were as follows: Vb1, Vr14, Sr24, Vr25, Vb1 + Sr24, Vb1+Vr25, Vr25+Vr14, Sr24+Vr25, Vb1+Sr24+Vr25, and one non-inoculated control. The design of combinations was based on the following six prioritized criteria: (1) PR solubilization abilities, (2) production of IAA (Magallon-Servin et al. 2019), (3) capacity to colonize maize roots (seedling experiment), (4) biocompatibility among strains, (5) biocompatibility with AM (Magallon-Servin et al. 2019), and (6) ability to form biofilm over PR surface. Two AM treatments were used: indigenous AM (iAM) and iAM with *Rhizophagus irregularis* (*Ri*) (iAM+*Ri*). The iAM treatment contained natural AM in the soil selected for this study. For the iAM+*Ri* treatment, half of the total soil was mixed homogenously with *R. irregularis* spores giving 750 spores kg^{-1} of soil.

Seed sterilization and inoculant preparation

Commercial maize seeds (*Zea mays* cv. Focus) were surface sterilized by washing them in 1.5 L of a 1% (vol/vol) of Tween 20 (Sigma-Aldrich) solution for 10 min to reduce the presence of pesticides that covered the seeds (Fludioxonil, Metalaxyl-M, and Azoxystrobin). The seeds were rinsed four times in dechlorinated sterile tap water, surface sterilized, and pre-germinated as described for the pouch experiments. Germinated seeds were submerged during 1 h in a 250-mL flask containing 160 mL of inoculum, prepared as described above. For multi-species inoculant, equal volume parts of each strain were used. In addition to the inoculation in suspension, immobilized inoculum was added to ensure plant colonization, since preliminary results showed that using only bacterial suspension did not render results. Thus, the bacteria were immobilized according to Bashan and de-Bashan (2015) with some modifications. Briefly, individual bacterial suspensions were prepared as described earlier. Then, 100 mL of the bacterial suspension was centrifuged (10,000×*g*), re-suspended in 20 mL of sterile saline solution, and mixed in 80 mL of a 2% (wt/vol) solution of alginic acid sodium salt from brown algae (Sigma-Aldrich). Alginate beads were produced by dripping the solution with a 5-cc syringe into a 2% (wt/vol) $CaCl_2$ solution, incubating 1 h to cure, and finally washing in sterile saline solution. For cell counting, beads were solubilized by immersing 1 g beads in 9 mL of 4% (wt/vol) solution of sodium bicarbonate ($NaHCO_3$) for 30 min at room temperature. Then, 10-fold serial dilutions were plated in TSA 10% (wt/vol) and incubated for 48 h at 28 °C. Fifty beads mL^{-1} of alginate-bacterial suspension solution contained 1×10^7 CFU g^{-1} of beads. In treatments that contained more than one PR-solubilizing bacterium, equal amounts of beads of each strain were mixed to give 1 g total.

Soil amendment with low-reactivity PR, seed inoculation, and plant growth determination

Approximately 1200 g of soil, air-dried, sieved to a 5-mm pore size, and containing 15% (vol/vol) perlite, was placed in a 15.4-cm diameter pot. P was added at a rate of 100 kg ha^{-1}; 65% of the recommended P was in the form of MPR (0.49 g pot^{-1}) and 15% as triple superphosphate (TSP) (0.034 g pot^{-1}). The TSP was used to avoid a severe P-starvation at the beginning of the experiment. Both P fertilizers were ground and mixed with the soil prior for the experiment. The last 20% of the P fertilizer was added as a 200-mL solution of KH_2PO_4 containing 16.02 mg L^{-1} to each pot two times per week starting at the fifth week after sowing.

Three germinated seeds were planted in each pot at 2-cm depth over 1 g of fresh immobilized cells (Fig. S1). Water was added daily as necessary to maintain soil moisture levels near field capacity. The pots were placed in a greenhouse under 16-h light at 25 °C and 8-h dark at 20 °C. After emergence, seedlings in each microcosm were thinned to one per pot. During the experiment, the plants received 200 mL two times a week of a solution of 14:0:14 fertilizers that contained 200 mg N L^{-1}.

At the end of the experiments, roots were used to determine the percentage of arbuscular mycorrhizal colonization (%AM) (Phillips and Hayman 1970). Shoots were dried at 65 °C for 72 h to determine dry weight. The total plant N, P, and K uptake were also determined. N was quantified by colorimetric procedure (Nkonge and Balance 1982) after plant material was digested in H_2SO_4–H_2SeO_3–H_2O_2 (Isaac and Johnson 1976). P and K were extracted by calcination; P was quantified by colorimetry (Murphy and Riley 1962), and K by atomic emission spectrometry (Perkin-Elmer 3300, Germany). The presence of potential phosphate-solubilizing bacteria (PPSB) was assessed too (Supplementary Material Table S2).

Statistical analysis

All experiments were repeated at least twice. All data were tested for normality and homogeneity of variance, and, when necessary, they were $\log_{10}$-transformed to better fit normalized distributions. Analysis of variance and comparison of treatment means including least significance difference, least squares means for the greenhouse experiments, or Tukey for the in vitro studies were conducted by general linear models of SAS 9.2 (Cari, NC: SAS Institute Inc.). Significance was set at $P < 0.05$.

Results

Biocompatibility

Results showed that none of the four selected PRSB inhibited the growth of each other. Therefore, we were able to test all the possible mixtures for the PR solubilization and biofilm determination assays and some selected mixtures for *in planta* tests based on the following criteria: (a) P solubilization capacity, (b) biocompatibility, (c) compatibility with mycorrhizae, and (c) different PGP-associated traits. Furthermore, the biocompatibility was also verified in plants during the growth-pouch experiment (see results below).

Phosphate rock solubilization, pH, and organic acid production of the four strains, individually and in combination

When cultured individually, the four PRSB showed different capacities to mobilize P from MPR after 7 days of incubation in liquid media (Fig. 1a) and to grow on the NBRIP-MPR medium as represented in the amount of protein (Fig. 1b, parameter of biomass produced). Vb1 released the highest amount of P (174 ± 3.63 mg P L^{-1}) and showed the highest P solubilization activity, 0.80 ± 0.05 μg of P μg^{-1} of protein among all the individual strains (Table 1, $P < 0.05$). Vr25 and Sr24 were the second-best PR solubilizers (127 ± 8.02 and 101 ± 2.8 mg P L^{-1}, respectively), but Vr25 statistically presented ($P < 0.05$) a higher value of P solubilizing activity (0.64 ± 0.01 μg of P μg^{-1} of protein) than Sr24 (0.42 ± 0.03 μg of P μg^{-1} of protein). Finally, Vr14 showed the lowest P value (68.6 mg P L^{-1}). However, Sr24 and Vr14 (0.38 ± 0.07 μg of P μg^{-1} of protein) presented statistically similar specific P-solubilizing activities.

Almost all mixed cultures containing Vb1 showed high levels of P solubilization (Fig. 1a, $P < 0.05$). The specific P solubilization activity of mixed cultures containing Vb1 ranged between 0.46 ± 0.03 and 0.78 ± 0.031 μg of P μg^{-1} of protein (Table 1). Among these mixed cultures, Vb1+Sr24+Vr25 and Vb1+Sr24 presented the highest levels of P immobilization: 270.33 ± 3.07 and 265.38 ± 27.3 μg of protein mL^{-1} (Fig. 1b) and a specific P solubilization activity of 0.62 ± 0.03 and 0.46 ± 0.03 μg of P μg^{-1} of

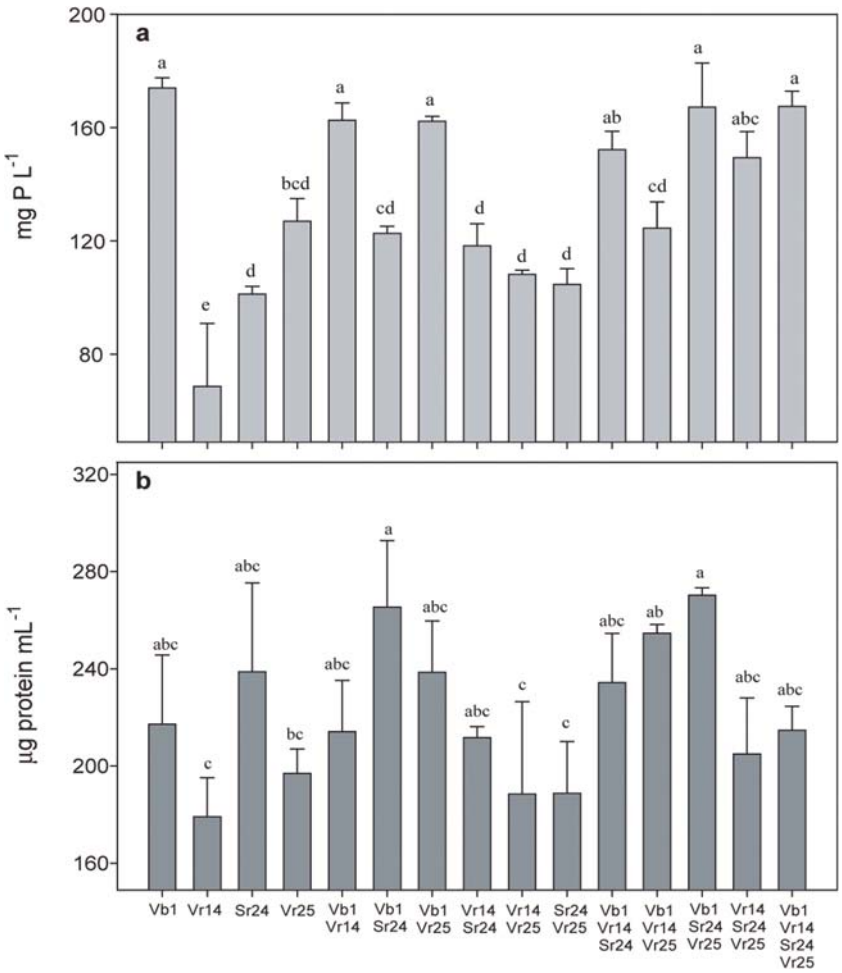

Fig. 1 PR solubilization by the different PRSB individually and in mixed culture (a) and bacterial growth (b) expressed as micrograms per protein per milliliter, after 7-day incubation in NBRIP-MPR liquid media. Error bars are + standard deviation ($n = 3$). Soluble P from non-inoculated control was subtracted from the respective treatments. Different letters indicate significant differences ($P < 0.05$) according to Tukey's test. Tested strains were *Asaia lannensis* (Vb1), *Pseudomonas* sp. (Vr14), *Rahnella* sp. (Sr24), and *Pantoea* sp. (Vr25)

Table 1 Effect of inoculation with phosphate rock-solubilizing bacteria (individually or in mixture) on the specific solubilization value of the culture filtrate of NBRIP-MPR, after 7 days of growth at 28 °C

Strain	μg of P μg^{-1} of protein
Vb1	0.808 ± 0.05^{a}
Vr14	0.382 ± 0.07^{b}
Sr24	0.429 ± 0.03^{b}
Vr25	0.644 ± 0.01^{cd}
Vb1+Vr14	0.764 ± 0.05^{a}
Vb1+Sr24	0.466 ± 0.03^{be}
Vb1+Vr25	0.683 ± 0.03^{acd}
Vr14+Sr24	0.557 ± 0.01^{cde}
Vr14+Vr25	0.59 ± 0.07^{cd}
Sr24+Vr25	0.559 ± 0.04^{de}
Vb1+Vr14+Sr24	0.651 ± 0.01^{cd}
Vb1+Vr14+Vr25	0.489 ± 0.02^{be}
Vb1+Sr24+Vr25	0.618 ± 0.03^{cd}
Vr14+Sr24+Vr25	0.737 ± 0.06^{ac}
Vb1+Vr14+Sr14+Vr25	0.781 ± 0.03^{a}

Data are means of the three replicates ($n = 3$). Statistical analysis was performed on the concentration of the specific P solubilization value (μg of P μg^{-1} of protein). In each row, lowercase letters represent significant differences at $P < 0.05$ according to Tukey test. Non-inoculated control did not show any solubilization. Tested strains were *Asaia lannensis* (Vb1), *Pseudomonas* sp. (Vr14), *Rahnella* sp. (Sr24), and *Pantoea* sp. (Vr25)

Table 2 Effect of inoculation with phosphate rock-solubilizing bacteria on the pH of the culture filtrate of NBRIP-MPR, after 7 days of growth at 28 °C

Strain	pH
Vb1	3.3^{a}
Vr14	4.0^{h}
Sr24	3.5^{efg}
Vr25	3.4^{bc}
Vb1+Vr14	3.3^{a}
Vb1+Sr24	3.6^{fg}
Vb1+Vr25	3.3^{a}
Vr14+Sr24	3.5^{def}
Vr14+Vr25	3.4^{b}
Sr24+Vr25	3.6^{g}
Vb1+Vr14+Sr24	3.5^{cd}
Vb1+Vr14+Vr25	3.5^{cde}
Vb1+Sr24+Vr25	3.4^{b}
Vr14+Sr24+Vr25	3.5^{cd}
Vb1+Vr14+Sr24+Vr25	3.4^{b}

Data are means of the three replicates ($n = 3$). Statistical analysis was performed on the concentration of H^{+} in solution and presented as pH. In each row, lowercase letters represent significant differences at $P < 0.05$ according to Tukey test. Non-inoculated control, pH = 7.0. Tested strains were *Asaia lannensis* (Vb1), *Pseudomonas* sp. (Vr14), *Rahnella* sp. (Sr24), and *Pantoea* sp. (Vr25)

protein, respectively (Table 1). The only mixed culture that solubilized P to a similar extent (no statistical differences) as Vb1 was the mixture of Vr14+Sr24+Vr25, which presented 149.3 ± 9.18 mg P L^{-1} of soluble P and a specific P solubilization activity of 0.73 ± 0.06 μg of P μg^{-1} of protein (Table 1).

After 7 days, bacterial growth on NBRIP-MPR was accompanied by an important decrease in the pH of the culture medium from an initial pH of 7 to 4 or less (Table 2). *A. lannensis* Vb1 and some of the mixed cultures containing this bacterium produced significantly more acidity than the other PRSB (Table 2). MPR solubilization was accompanied by the presence of two main OAs: 2-ketogluconic and/or D-gluconic (Fig. 2); however, there were other OAs detected on the analysis but they represented less than 10% of the total organic acid production. D-Gluconic acid seemed to be involved in the mobilization of P from MPR by Sr24 and those mixtures containing it (Fig. 2a). Conversely, MPR solubilization by Vb1, Vr14, and Vr25 was accompanied mainly by the production of 2-ketogluconic acid (Fig. 2b) compared to Sr24. Although Vb1 and Vr25 did not produce D-gluconic acid when used individually, the mixture of Vb1+Vr25 produced 253.7 ± 34.29 mg 100 mL^{-1} of D-gluconic acid in addition to 2-ketogluconic acid (Fig. 2a, b).

Determination of biofilm formation capacity on abiotic surfaces and over MPR surface

Determination of the Specific Biofilm Index by the traditional crystal violet method (Fig. 3) showed significant differences when the strains were used individually and in mixture. Vr14 presented the highest SBF values, followed by Sr24 and Vr25 (both strains presented similar Specific Biofilm Index values). Finally, Vb1 presented the lowest SBF values compared to the other three strains (Fig. 3). When cultured in combination, those associated with Vr14, except Vr14+Sr24+Vr25, presented statistically similar SBF values as Vr14 when used individually. The remaining combinations presented no significant differences compared to Vb1, Sr24, and Vr25 used individually (Fig. 3).

Determination of the biofilm formation capacity over MPR particles, measured as the production of extracellular polysaccharides (Fig. 4a), produced results different from those obtained by the classic crystal violet method (Fig. 3). The capacity of Vr14 (0.023 μg mL^{-1}) to produce biofilm over MPR particles was significantly lower than Sr24 (0.052 μg mL^{-1}); Vr14 produced a statistically similar concentration of extracellular polysaccharides as Vb1 (0.018 μg mL^{-1}) and Vr25 (0.040 μg mL^{-1}) when used individually. The mixed cultures Vr14+Vr25, Vb1+Sr24, and the four strains together produced the highest quantities of extracellular polysaccharides among all the treatments (Fig. 4a).

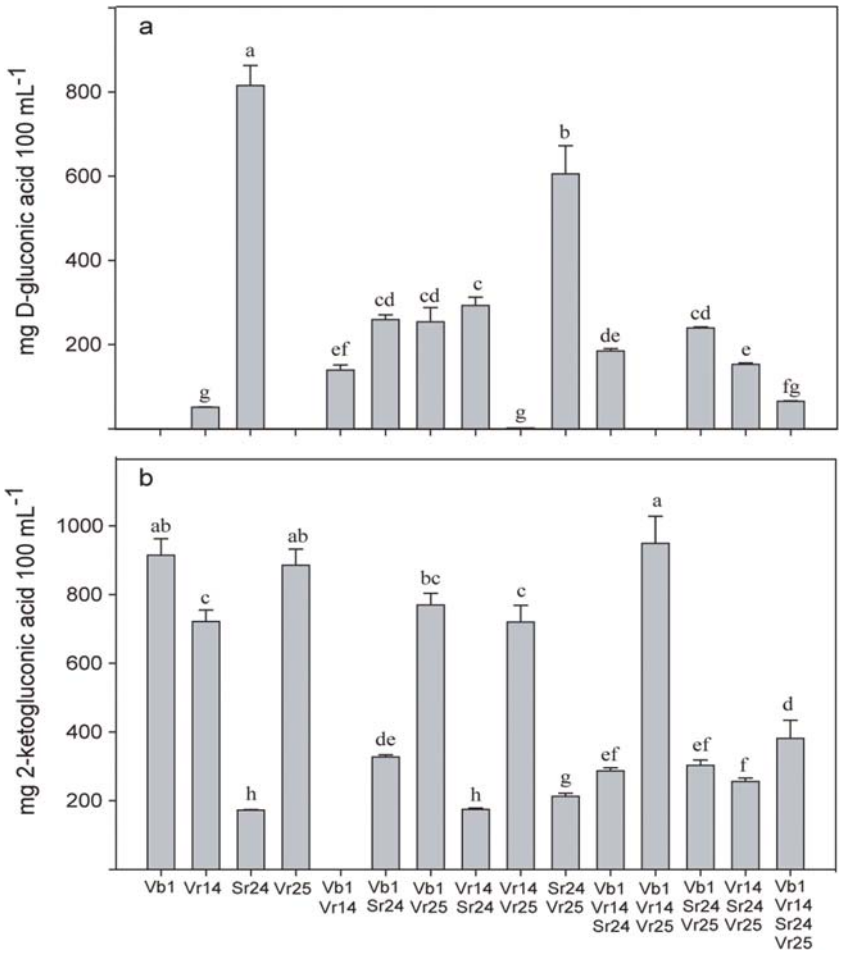

Fig. 2 Production of organic acids by the selected PRSB used individually and in mixture: (a) D-gluconic acid and (b) 2-ketogluconic produced by the PRSB isolates after 7 days of incubation in liquid cultures. Error bars are + standard error ($n = 3$). Different letters show significant differences by Tukey's test ($P < 0.05$). Tested strains were *Asaia lannensis* (Vb1), *Pseudomonas* sp. (Vr14), *Rahnella* sp. (Sr24), and *Pantoea* sp. (Vr25)

Evaluation of the PR solubilization over MPR particles showed that Vb1, Vr14, and Vr25 presented higher values of soluble P (81.9, 74.79, and 64.28 mg L^{-1}, respectively) than the Sr24 treatment (33.68 mg L^{-1}) used individually (Fig. 4b). The MPR solubilization was accompanied by a decrease in pH values (Table 3) compared to the uninoculated treatment (pH

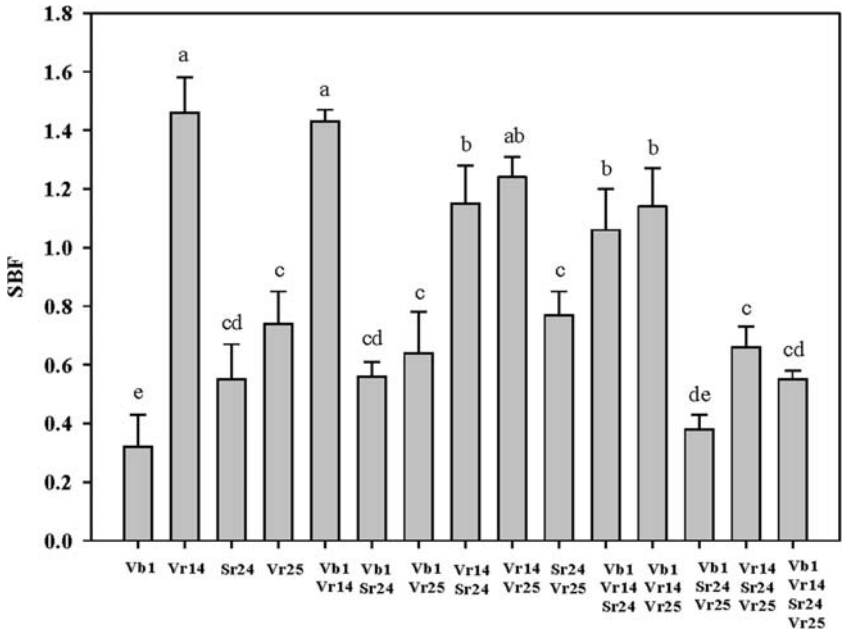

Fig. 3 Specific biofilm formation index determined by the traditional crystal violet method of the selected PRSB used individually and in combination. Error bars are + standard deviation ($n = 5$). Non-inoculated control values were subtracted from the respective treatments. Different letters show significant differences by Tukey's test ($P < 0.05$). Tested strains were *Asaia lannensis* (Vb1), *Pseudomonas* sp. (Vr14), *Rahnella* sp. (Sr24), and *Pantoea* sp. (Vr25)

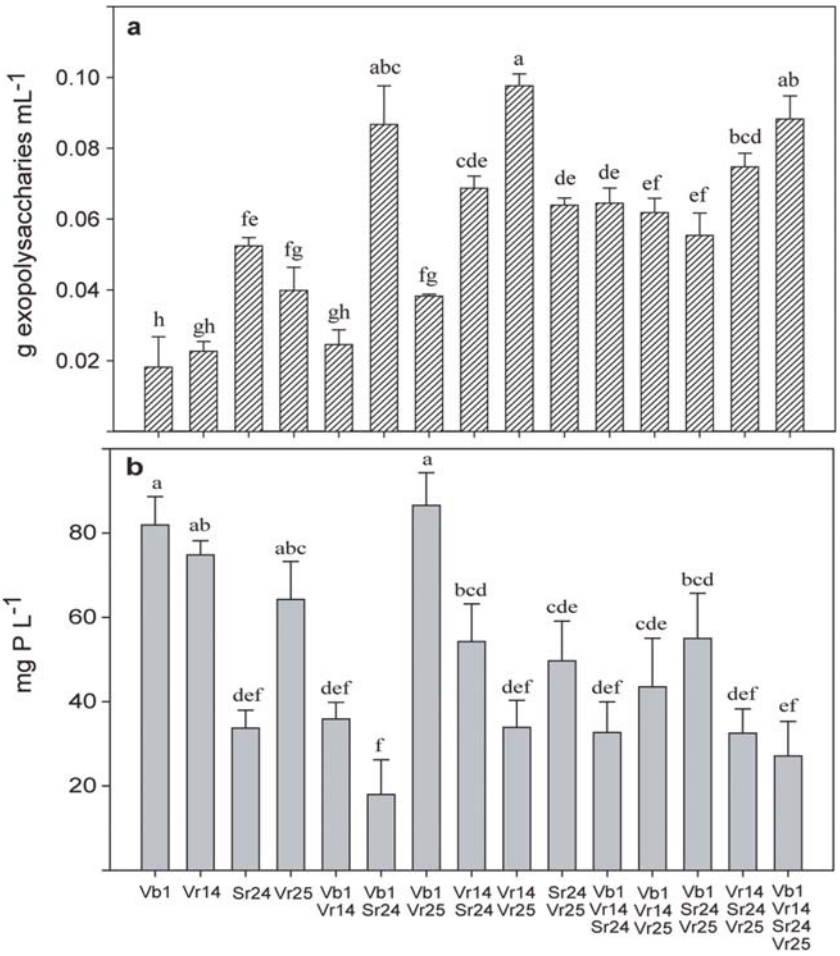

Fig. 4 Amount of (a) exopolysaccharides produced and (b) P solubilized by the different PRSB isolates used individually or in mixture. Error bars are + standard deviation ($n = 3$). Non-inoculated control values were subtracted from the respective treatments. Significant differences according to the Tukey test, at $P < 0.01$ for exopolysaccharides and $P < 0.05$ for phosphorus, are indicated by different letters. Tested strains were *Asaia lannensis* (Vb1), *Pseudomonas* sp. (Vr14), *Rahnella* sp. (Sr24), and *Pantoea* sp. (Vr25)

7.0). However, acidification of the media was not related to the capacities of the selected isolates to dissolve MPR in the biofilm. For example, Sr24 and Vr25 individually presented similar pH values but presented different levels of P-solubilization (33.68 and 64.28 mg L^{-1}, respectively) (Fig. 4b).

In mixture, Vb1+Vr25 showed the highest P level (86.52 mg L^{-1}) and presented the lowest pH value (3.3) (Fig. 4b and Table 3). Furthermore, Vb1+Sr24 presented the lowest soluble P level (18 mg L^{-1}) and pH similar to the other mixtures. As a general tendency, all mixtures (with the exception of Vb1+Vr25) presented similar P solubilization and pH reduction regardless of the PRSB used (Fig. 4b).

Based on our results, the biofilm formation capacity (determined as the extracellular polysaccharides production) was not related to the PR solubilization capacity. Vb1 showed a low biofilm formation capacity, but it was a good PR-solubilizing strain. In contrast, Sr24 showed a good biofilm formation capacity, but did not mobilize as much P from MPR in the medium as the rest of the strains when used individually. Among the selected PRSB, *Pantoea* sp. Vr25 was as good at forming biofilms as Sr24 and was able to dissolve more P from MPR than Sr24 (Fig. 4b). Mixing the selected PRSB did not improve MPR solubilization over individual treatments. In addition, mixtures better at biofilm formation than their respective individual treatments were not better at mobilizing MPR.

Effect of the PRSB on growth and root colonization of seedlings in growth pouches

Rahnella sp. Sr24 showed a significantly higher effect on the growth of 2-week-old maize seedlings compared to the uninoculated control (Table 4). When this strain was mixed with other strains like Vb1, Vr25, or Vr14, the shoot dry weight was significantly higher than the uninoculated control. The effect of Sr24 and the mixtures containing it on the root dry weight showed a similar trend compared with the uninoculated control, however, used individually and in the mixtures Vb1+Sr24+Vr25, Vb1+Sr24, and Vb1+Vr25 produced significantly more total dry weight than the uninoculated control.

A. lannensis Vb1 and *Pseudomonas* sp. Vr14 did not colonize the roots as well as *Rahnella* sp. Sr24 and *Pantoea* sp. Vr25, either individually or in mixtures (Fig. 5). Individually, Vb1 and Vr14 showed lower CFU gr-1 dry roots than Sr24 and Vr25, which demonstrates a lower capacity to colonize roots under the experimental conditions. However, when mixed (Vb1+Vr14), the Vr14 strain appeared to be a better

Table 3 Effect of inoculation with phosphate rock solubilizing bacteria on the pH of the culture filtrate when grown over MPR particles, after 5 days at 28 °C

Strain	pH
Vb1	3.49^{b}
Vr14	3.51^{bc}
Sr24	3.85^{de}
Vr25	3.73^{cd}
Vb1+Vr14	3.85^{de}
Vb1+Sr24	4.43^{e}
Vb1+Vr25	3.33^{a}
Vr14+Sr24	3.81^{de}
Vr14+Vr25	4.05^{de}
Sr24+Vr25	3.81^{de}
Vb1+Vr14+Sr24	3.95^{de}
Vb1+Vr14+Vr25	4.19^{de}
Vb1+Sr24+Vr25	3.78^{de}
Vr14+Sr24+Vr25	3.98^{de}
Vb1+Vr14+Sr24+Vr25	3.9^{de}

Data are means of the three replicates ($n = 3$). Statistical analysis was performed on the concentration of H^+ in solution and presented as pH. In each row, lowercase letters represent significant differences at $P < 0.05$ according to Tukey test. Non-inoculated control, pH = 7.0. Tested strains were: *Asaia lannensis* (Vb1), *Pseudomonas* sp. (Vr14), *Rahnella* sp. (Sr24), and *Pantoea* sp. (Vr25)

root colonizer than Vb1. The rest of the mixtures, including Sr24 and Vr25, were good root colonizers (Fig. 5) under the experimental conditions.

Table 4 Effect of inoculation with PRSB (used individually or in mixed culture) on dry matter yields of 2-week-old maize seedlings

Dry weight (mg)*			
Treatment	Shoots	Roots	Total
A. lannensis Vb1	214.7^{abc}	109.0^{cd}	324.0^{bcd}
Pseudomonas sp. Vr14	204.7^{bcd}	105.2^{d}	310.0^{bcd}
Rahnella sp. Sr24	240.9^{a}	135.7^{a}	376.6^{a}
Pantoea sp. Vr25	206.6^{bcd}	105.7^{d}	312.3^{bcd}
Vb1+Vr14	203.3^{bcd}	113.8^{bcd}	317.1^{bcd}
Vb1+Vr25	207.6^{bcd}	127.1^{abc}	334.8^{abc}
Vb1+Sr24	219.0^{ab}	117.1^{abcd}	336.2^{abc}
Vb1+Vr14+Vr25	190.9^{cd}	116.6^{abcd}	307.6^{dc}
Vr14+Sr24+Vr25	221.4^{ab}	130.0^{ab}	351.4^{ab}
Non-inoculated control	181.4^{d}	100.9^{d}	282.4^{d}
LDS ($P < 0.05$)	27.3	19.1	43.3

Values are means (mg) of the three replicates (n = a rack containing 7 growth pouches). Tested strains were *Asaia lannensis* (Vb1), *Pseudomonas* sp. (Vr14), *Rahnella* sp. (Sr24), and *Pantoea* sp. (Vr25)

*Dry weight is expressed in mg per growth pouch containing 2 seedlings

Greenhouse experiment

Inoculation with the PRSB (individually and in combination) increased significantly the iAM colonization of maize roots compared to the uninoculated control (Fig. 6a). When used individually, *Pseudomonas* sp. Vr14 and *Pantoea* sp. Vr25 affected positively the establishment of iAM in maize roots compared to *A. lannensis* Vb1 and *Rahnella* sp. Sr24. Furthermore, maize roots inoculated with the combination of Vr14 and Vr25 had 1.6 times higher iAM% than the uninoculated control (Fig. 6a). Also, the combination of Vb1 and Sr24 resulted in a higher percentage of iAM than either strain used individually. Even though the natural mycorrhizal potential of the soil used was high, plant inoculation with the selected PRSB (used either individually or in combination) increased the extent of colonization by the iAM over the uninoculated control.

Inoculation with the selected PRSB and *Rhizophagus irregularis* significantly affected the percentage of AM root colonization (%AM) in maize plants. The control that received neither bacterial nor *R. irregularis* inoculation was colonized by indigenous mycorrhizae (iAM) and presented 47.71% of AM colonization of maize roots 10 weeks after planting (Fig. 6a). This percentage of AM root colonization was significantly increased to 76.18% by the incorporation of *R. irregularis* inoculum (Fig. 6b). The percentage of AM root colonization was more affected by the introduction of *R. irregularis*, than by the inoculation with the PRSB. In fact, only the percentages of AM root colonization of Vr14+*Ri*, Vr25+*Ri*, and Vr14+Vr25+*Ri* and Sr24+Vr25+*Ri* treatments were significantly higher than those of plants that were inoculated only with *R. irregularis*. Additionally, inoculation of PRSB, with and without *R. irregularis*, significantly enhances the number of potential phosphate solubilizer bacteria found in maize roots (Supplementary Material Table S2).

Plant inoculation with the PRSB used individually in the absence of *R. irregularis*, produced significantly higher dry weight than the non-inoculated control, except with Vb1 (Table 5). However, significantly higher dry weight was observed only by inoculating plants with *Rahnella* sp. Sr24 and *Pantoea* sp. Vr25, resulting in 1.4- and 1.5-fold increases, respectively, over the uninoculated control. It is noteworthy that in the absence of *R. irregularis*, maize growth was significantly greater when plants were inoculated with PRSB mixtures, regardless of the bacterial components in the mixture (Table 5). The incorporation of *R. irregularis* in the PRSB inoculation produced different results. When used individually in addition to the *R. irregularis*, most strains showed no significant differences compared to when used in mixture. However, the highest overall growth, with and without the incorporation of *R. irregularis*, was observed in the mixed inoculants containing Vr25+Vr14+*Ri* and Sr24+Vr25+*Ri*.

Plant N content ranged from 51.29 to 93.7 mg N plant^{-1}, and 76.28 and 98.70 mg N plant^{-1} in the non-inoculated and

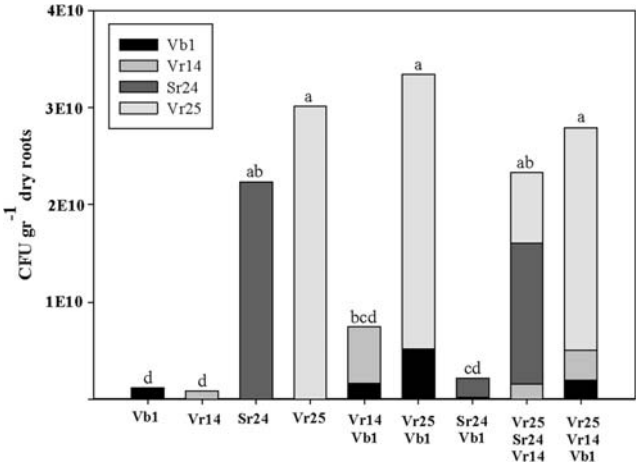

Fig. 5 Root colonization of 2-week-old maize seedlings by the PRSB. Values (CFU g^{-1} of dry roots) are means of $n = 3$. Different letters indicate significant ($P < 0.05$) differences based on Tukey's test. 1E10 equals 1×10^{10} CFU g^{-1} dry roots. Tested strains were *Asaia lannensis* (Vb1), *Pseudomonas* sp. (Vr14), *Rahnella* sp. (Sr24), and *Pantoea* sp. (Vr25)

R. irregularis treatments, respectively (Table 5). The N uptake was significantly increased by PRSB inoculation compared to the non-inoculated control in the absence of *R. irregularis*.

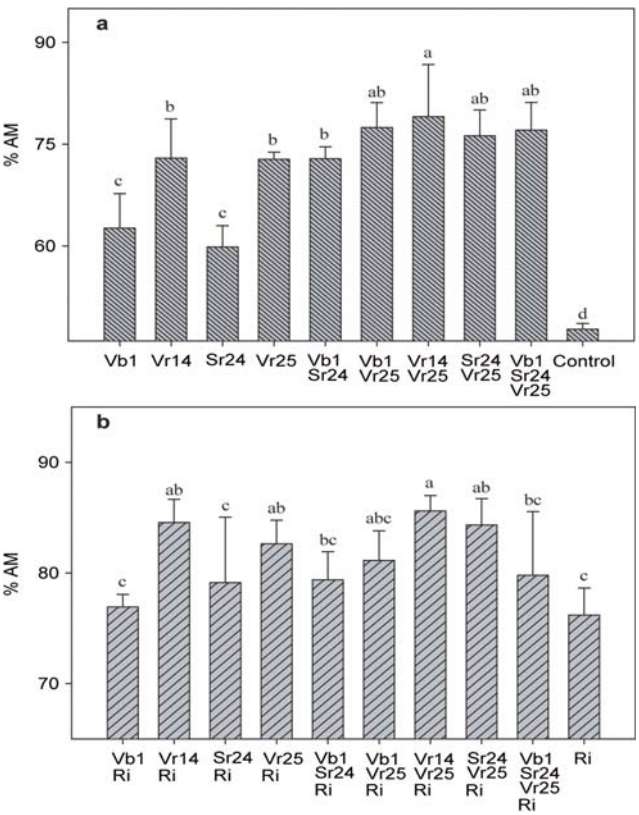

Fig. 6 Effect of PRSB inoculation (used individually or in mixture) on the percentage root mycorrhization by the (a) iAM and (b) iAM+*Ri* treatment. Error bars are + standard deviation ($n = 4$). Different letters indicate significant ($P < 0.05$) differences based on LS means test. Tested strains were *Asaia lannensis* (Vb1), *Pseudomonas* sp. (Vr14), *Rahnella* sp. (Sr24), and *Pantoea* sp. (Vr25)

Moreover, inoculation with the mixtures significantly increased the total N uptake over the strains used separately. Inoculation of maize plants with only *R. irregularis* significantly increased the plant N uptake, but when PRSB were used in combination with *R. irregularis* (whether the strains were used individually or in mixture), the N uptake remained similar to the *R. irregularis*-only treatment. Plant P content ranged from 4.07 to 7.38 mg P $plant^{-1}$, and 4.71 and 6.73 mg P $plant^{-1}$ in the non-inoculated and *R. irregularis* treatment, respectively (Table 5). Plant P uptake followed a trend similar to that of N. The P uptake was significantly greater with the mixed inoculants than with each strain individually. P uptake was not enhanced by the incorporation of *R. irregularis* into the PRSB inoculation (Table 5). Finally, plant K content ranged from 67.84 to 111 mg K $plant^{-1}$, and 90 and 119.08 mg K $plant^{-1}$ in non-inoculated and *R. irregularis* treatments, respectively (Table 5). Inoculation with the PRSB used individually and in mixture increased K uptake in a significant manner compared to the non-inoculated control in the absence of *R. irregularis*. Incorporation of *R. irregularis* into PRSB inoculation (used individually or in mixture) did not enhance plant K uptake over the *R. irregularis*-only treatment. In all, increases in plant N, P, and K uptake of 13.08%, 26.50%, and 13.9%, respectively, over the *R. irregularis*-only treatment were observed after inoculation with the Vr25+Vr14+*Ri* mixture (Table 5).

Discussion

It is challenging to formulate a multifunctional microbial inoculant by adding appropriate microbial combinations to apply to plants to improve their growth (Pandey et al. 2012). However, due to the complexity of the soil microbial ecology and the diversity of niches in the rhizosphere environment, a mixture of biocompatible bacteria all able to solubilize PR but with different PGP traits enhances the chance that different microorganisms (not only different strains) occupy different niches. Although all selected PRSB were able to solubilize phosphorus, our results suggest that the mix of the strains is more effective on solubilization of low-reactivity PR, than each strain alone. This is especially true when we consider the P immobilization determined indirectly by bacterial biomass assessed as protein. It is recognized that P contained in the microbial biomass plays an important role in plant available P in soils (Chen et al. 2000). Furthermore, by including different PRSB for an inoculant formulation, we expand the possibilities of each strain acting in a different P-plant acquisition strategy. For example, Sr24, Vr25, and Vr14 have been reported to produce IAA, phosphatases, and/or phytases, are good colonizers of *Rhizophagus irregularis* hyphae, and are able to solubilize P from PR with different reactivities (Magallon-Servin et al. 2019). The contribution of

Table 5 Effect of inoculating maize plants with PRSB, either individually or in different combinations, in a soil amended with MPR in the presence and absence of *Rhizophagus irregularis* (*Ri*) under greenhouse conditions

iAM	Dry weight (g)	N uptake (mg)	P uptake (mg)	K uptake (mg)
Non-inoculated	3.3^{d}	51.29^{f}	4.07^{f}	67.84^{d}
Vb1	4.3^{d}	69.36^{e}	4.88^{ef}	91.45^{bc}
Vr14	4.4^{cd}	68.9^{e}	5.62^{bcde}	92.01^{bc}
Sr24	4.9^{c}	81.2^{bcde}	6.21^{abcde}	105.05^{abc}
Vr25	5.0^{c}	73.51^{e}	6.5^{abcd}	91.7^{bc}
Vb1+Sr24	6.0^{abc}	93.7^{abc}	7.38^{a}	111.0^{abc}
Vb1+Vr25	5.9^{abc}	82.36^{abcde}	6.47^{abcd}	104.78^{abc}
Vr25+Vr14	6.1^{abc}	85.9^{abcd}	7.26^{a}	109.62^{abc}
Sr24+Vr25	5.1^{c}	84.85^{abcde}	7.14^{ab}	91.46^{abc}
Vb1+Sr24+Vr25	5.3^{bc}	89.44^{abcd}	7.08^{abc}	109.92^{abc}
Ri	6.1^{abc}	87.28^{abcd}	5.32^{def}	105.34^{abc}
Vb1+*Ri*	6.4^{abc}	91.01^{abcd}	5.99^{abcde}	112.40^{ab}
Vr14+*Ri*	5.0^{c}	77.18^{cde}	4.83^{ef}	97.02^{abc}
Sr24+*Ri*	6.4^{ab}	97.30^{ab}	6.62^{abcd}	117.65^{a}
Vr25+*Ri*	6.4^{abc}	91.28^{abcd}	5.87^{abcde}	113.60^{a}
Vb1+Sr24+*Ri*	5.4^{abc}	76.28^{de}	4.72^{ef}	90.065^{c}
Vb1+Vr25+*Ri*	5.7^{abc}	86.87^{abcd}	5.58^{cdef}	104.44^{abc}
Vr25+Vr14+*Ri*	6.6^{ab}	98.70^{a}	6.73^{abcd}	119.087^{a}
Sr24+Vr25+*Ri*	6.6^{a}	93.72^{abc}	6.17^{abcde}	112.36^{ab}
Vb1+Sr24+Vr25+*Ri*	5.6^{abc}	79.42^{cde}	4.71^{ef}	99.57^{abc}

Values are means of the four replicates. In the same column, significant ($P < 0.05$) differences are indicated by different letters according to the LS means test

rhizobacteria in the P-acquisition strategies of the plant is complex and involves different mechanisms. As observed, by mixing the PR-solubilizing and PGP abilities of different strains, the potential impact of each component on P nutrition could be increased.

The production of organic acids and the release of protons is a well-recognized mechanism of P-solubilizing rhizobacteria. In general, organic acids release P from the phosphate mineral by proton substitution for Ca^{+2} (Rodriguez and Fraga 1999). Bacteria having a P-solubilizing phenotype can dissolve highly insoluble phosphates such as PR ore due to the low pK_a(s) of the glucose oxidation products gluconic acids (~ 3.4) and 2-ketogluconic acid (~ 2.6). However, our results do not allow determining which one is associated with a higher solubilization of MPR. Thus, *Rahnella* sp. Sr24 and *Pantoea* sp. Vr25 released similar quantities of P in the medium and presented no difference in proton release, but they produced different organic acids. Furthermore, *A. lannensis* Vb1 and *Pantoea* sp. Vr25 used individually did not produce D-gluconic acid, but when mixed, we detected D-gluconic and 2-ketogluconic acids in the supernatant after 7 days of incubation. The supernatant of this mixture contained quantities of soluble P similar to that of Vb1 growing alone. Goldstein (2007) mentioned that oxidation of glucose to gluconic acid is common among P-solubilizing bacteria, and a second periplasmic oxidation of gluconic acid to 2-ketogluconic acid via gluconate dehydrogenase is also common in rhizobacteria presenting a P-solubilizing phenotype. Hence, accumulation of both organic acids when Vb1 and Vr25 are mixed might result from a metabolic change in the direct oxidation of glucose when these strains are cultured together.

Biofilm formation capacity has been related to the ability of rhizobacteria to colonize the roots and soil environment, and there has been interest in developing biofilms by mixing P-solubilizing microorganisms to increase mobilized P from low-solubility P sources (Seneviratne et al. 2008). Our findings show that all strains were able to develop biofilms on abiotic surfaces. When the biofilm formation capacity was assessed by the crystal violet method, our results showed that *Pseudomonas* sp. Vr14 and its associated mixtures developed stronger biofilms compared to the rest of the strains. There are several *Pseudomonas* sp. isolated from the rhizosphere that have been characterized as a strong-biofilm forming bacteria when evaluated with the traditional crystal violet method, such as *Pseudomonas fluorescens* FAP2 (Ansari and Ahmad 2019a), *P. stutzeri* A1501 (Wang et al. 2017), *P. azotoformans*, *P. entomophila*, and *P. endophytica* (Ansari and Ahmad 2019b). Conversely, *Rahnella* sp. Sr24 and *Pantoea* sp. Vr25 (used individually) produced stronger biofilms over MPR particles compared to *Pseudomonas* sp. Vr14 when determined by extracellular polysaccharides quantification. Extracellular polysaccharides play a key function in the initial attachment of cells to different substrates and protection against environmental stress and dehydration (Gupta et al. 2017). Determining biofilm formation by measuring total

extracellular polysaccharides has been reported as a good method to estimate the biofilm mass attached to PR surface (Vandevivere and Kirchman 1993; Liu et al. 1994; Ueshima et al. 2004). For a PR-solubilizing inoculant, the capacity to form biofilm over PR particles and rhizosphere is relevant. In agreement, Taktek et al. (2017) found that two hyphobacteria, *B. anthina* Ba8 and *R. miluonense* Rm3, formed strong biofilms over PR and hydroxyapatite when using NBRIP medium, and they were able to effectively mobilize P. The differential capacities of the PRSB bacteria to form biofilms and solubilize PR particles could be influenced by a number of factors, including the mineral composition (accessory minerals) of PR particles. Jones and Beent (2017) suggested that the biofilm and extracellular polysaccharides production are influenced by nutrient content of the growth medium and phosphate limitations in multi-specific biofilms when different rocks/minerals were used as an abiotic surface to form biofilms. They observed that those minerals that contained insoluble P (limestone, dolostone, basalt) had a significantly higher biofilm biomass. Ghosh et al. (2019) observed that the P concentration on different PR was relevant for the biofilm of *Burkholderia tropica* and *B. unamae* over insoluble phosphate granules. In our results, *Rahnella* sp. Sr24 and *Pantoea* sp. Vr25 produced stronger biofilms compared to *Asaia lannensis* Vb1 and *Pseudomonas* sp. Vr14, although all bacteria presented comparable capacities to solubilize PR in planktonic cultures. These differential results about biofilm formation can be attributed to the fact that under stress conditions (no soluble P), bacteria tend to form stronger biofilms compared with high nutrient concentration. Environmental stressors, such as temperature, salinity, and water stress, can affect biofilm formation in certain rhizobacteria, such as *Pseudomonas* sp., *Pantoea* sp., and *Bacillus* sp. (Ansari and Ahmad 2019b). Metal resistance is also related to the biofilm formation capacity, especially when high-rich minerals like PRs are used as an abiotic surface. Harrison et al. (2007) reviewed how the multimetal resistance is related to the extracellular polysaccharide production and the biofilm formation in different bacteria. Metals act as stressors that can lead to enhancement of metal resistance in certain bacteria. We observed that those strains that showed higher biofilm formation over PR particles (Sr24 and Vr25) presented higher minimal inhibitory concentration metal resistance toward Zn+2 and Mn+2 compared to Vb1 and Vr14, and higher minimal inhibitory concentration values toward Fe+3 and Al+3 compared to Vr14. When inoculated in mixture, certain combinations, such as Vr14+Vr25 and Vb1+Sr24, improved biofilm formation compared to monoculture strains. This aspect is highly relevant when screening for PR-solubilizing mixtures. Since biofilms tend to resist environmental stresses better than free cells, they could be used for more successful establishment of introduced microorganisms when used as inoculants (Seneviratne et al. 2008).

Evaluation of the inoculation effects of the strains used individually or in mixture on the growth of maize seedlings showed that *Rahnella* sp. Sr24 significantly increased the dry weight of roots and shoots of 2-week-old maize seedlings. Some mixtures containing Sr24 showed the same tendency. Because this experiment was performed under unlimited-P conditions, we could attribute the positive effect of Sr24 to causes other than its P-solubilizing capacity, such as its capacity to produce IAA. This step was relevant to select those mixtures to test under greenhouse conditions, even though *Rahnella* sp. Sr24 did not stand out as a PR solubilizer. Compared to Vb1, it had a relevant impact on the growth of maize seedlings based on other PGP traits.

Determination of root colonization by the selected PRSB isolates showed that *Pseudomonas* sp. Vr14, a strong biofilm forming bacteria, was not a competitive root colonizer compared to *Pantoea* sp. Vr25 or *Rahnella* sp. Sr24, which presented a lower biofilm formation capacity determined by the traditional crystal violet method. Nevertheless, those two strains formed stronger biofilms over MPR particles compared to Vr14 and Vb1. Based on our results, Vr25 was a very competitive strain when used with other PRSB. This strain was previously characterized as presenting the highest swimming motility phenotype among the isolates (Magallon-Servin et al. 2019). Other studies have established that motility is a key trait in root colonization by rhizobacteria (Barahona et al. 2010). For example, the non-motile *P. putida* PaW8 presented significant lower attachment to wheat roots than the motile *P. putida* PaW8 in competitive assays, suggesting that motility enables cells to reach attachment sites more rapidly or increases the chances of finding potential binding sites (Turnbull et al. 2001). Similar results have been reported for *Pseudomonas fluorescens* in tomato root-tip colonization (de Weert et al. 2002) and for *P. pseudoalcaligenes* AVO110 in avocado roots (Pliego et al. 2008). Other results have proven that motility, rather than forming mature biofilms on abiotic surfaces, is a key trait for competitive root colonization in *P. fluorescens* F113 (Barahona et al. 2010).

The greenhouse results showed that all mixes of PRSB increased dry weight, and nutrient (N, P, and K) uptake, and compared each strain individually in the absence of *R. irregularis*. The beneficial effects of *Rahnella* sp. Sr24 and *Pantoea* sp.Vr25, and *Pseudomonas* sp. Vr14 in plant growth and nutrient uptake cannot be attributed only to their ability to solubilize PR. These strains presented other PGP-associated traits associated with plant P nutrition, such as the production of IAA, siderophores, phosphatases, and/or phytases. Moreover, all are good hyphae colonizers of *R. irregularis* (Magallon-Servin et al. 2019), presented a beneficial effect on percentage AM root colonization, and were biocompatible with each other. Therefore, as we hypothesized, the mixtures of strains, regardless of their performance in PR-solubilization in planktonic cultures, proved to be a

good multi-species inoculant that improved plant P nutrition and growth under low-P soils when amended with low-reactive PR.

Inoculation with *Rhizophagus irregularis* in combination with the strains affected further the maize plant response. Babana and Antoun (2006) reported that dual inoculation of wheat plants with *Aspergillus awamori* C1 or *Penicillium chrysogenum* C13 (P-solubilizing microorganisms) in addition to *Glomus irregulare* resulted in a positive interaction that increased shoot yield, and a combination of the same *A. awamori* C1+ *Gi* + *Pseudomonas* sp. Br2 increased the P concentration of wheat grain in soils amended with PR, suggesting that P solubilization is an important mechanism in growth promotion. Taktek et al. (2015) proved the interaction between PSB and *Rhizophagus irregularis* extraradical hyphae affected the solubilization of PR. They observed that the AM extraradical hyphae are an active distributor of fresh plant C to soil microbes, enhancing PR solubilization through this interaction. Our results support the hypothesis that by combining PRSB and AM, it is possible to increase plant growth, and that *R. irregularis* interaction with rhizobacteria is relevant. However, when choosing the PSB to interact with AM (multi-species inoculant), the in vitro P-solubilizing capacity cannot be the only factor taken into account; it is necessary to consider other PGP traits and biocompatibility. Wu et al. (2005) reported that maize inoculation with *Azotobacter chroococcum* (N-fixer), *Bacillus megaterium* (P solubilizer), *Bacillus mucilaginous* (K solubilizer), and AM fungi under low fertilization levels increased not only root colonization by *G. mosseae* and *G. irregulare* but plant growth and P uptake. In other studies, Battini et al. (2017) showed the beneficial effect on growth and phosphorus uptake (assessed by radioactive P uptake) in maize by mycorrhizosphere bacteria (*Streptomyces* sp.), providing evidence of the positive interaction between AM and mycorrhizosphere bacteria. We observed that inoculation of maize by combining more than one strain of PRSB improved the nutrient uptake and percentage of iAM colonization over single inoculation. Furthermore, adding *R. irregularis* into the mixture improved the results. In general, our findings support the idea that bacterial combinations can enhance plant growth more efficiently than single species inoculations, because each strain presents different PGP-associated traits that together act synergistically on behalf of the plant. In addition, AM fungi has an effect on plant nutrient uptake because it has the ability to be associated with roots, therefore extending the soil volume that plants are able to explore for P or N uptake (Toro et al. 1997).

Conclusions

A common way to select PR-solubilizing bacteria is by quantifying the mobilized P from low-soluble P sources. However, studies rarely evaluate combinations of PRSB on low-reactivity PR in order to select those with similar or better PR-biosolubilization than individual cultures. Based on our results, we concluded that combining different biocompatible PRSB isolates enhanced significantly growth and nutrient uptake in maize plants in comparison to monoculture inoculant. Further, combining the use of mixed PRSB with AM proved to increase shoot dry weight, nutrient uptake, and the percentage of indigenous AM root colonization in maize, highlighting the potential for their use for low-input agriculture in low-P soils amended with PR. Interestingly, although the biofilm formation capacity was not directly reflected in PR solubilization, those bacteria with higher biofilm formation showed higher resistance to heavy metals, and higher successful root colonization, giving them an edge as growth promoters. In summary, a successful multi-species PRSB inoculant should comply with a multi-criteria selection based on (1) high capacity to solubilize different phosphate rock, (2) high biocompatibility, (3) compatibility with AM, (4) good biofilm formation capacity over PR and roots, and (4) growth-promoting traits.

The fate of the inoculated microorganisms was not monitored during the greenhouse experiment, due to the complexity to develop a method that will allow us to track each strain effectively in the rhizosphere/rhizoplane or on the hyphae of *R. irregularis*. However, we push forward the development of new approaches to follow the inoculation of multiple-species consortia.

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